

Comparative functional role of PC7 and furin in the processing of the HIV envelope glycoprotein gp160

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Abstract The intracellular proteolytic processing of HIV envelope glycoprotein gp160 into gp120/gp41 is an essential step for virus infectivity. Several convertases, belonging to the pro-protein convertase family, have been proposed as candidate gp160 processing enzymes. Here we demonstrate using RT-PCR that resting human T4 lymphocytes weakly express PC7, furin, and PC5 mRNA whereas lymphocytes activated under conditions favoring HIV replication express 5–10-fold higher levels of furin and PC7. In this report, we examined the capability of the newly cloned convertase PC7 to cleave gp160 into gp120/gp41 and compared it to furin. This was carried out in a cell-based assay whereby both gp160 and the cognate convertase were co-expressed in the constitutively secreting BSC40 cells and in the regulated AtT20 cells, as well as using two in vitro assays which examined the cleavage of gp160 or of a synthetic peptide spanning the cleavage site. The data demonstrate that PC7 can cleave specifically and in a cell-type specific manner gp160 into gp120/gp41, suggesting that both furin and PC7 are so far the major PC-like candidate gp160 convertase in T4 lymphocytes.

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Key words: Furin; PC7; HIV; gp160; gp120; gp41; Convertase; Processing

1. Introduction

The lymphotropic HIV enveloped virus infects target cells by a multistep process, involving both fixation to the CD4⁺ receptor and fusion between the viral envelope and host cell membrane. Fusion is mediated by the mature envelope glycoprotein gp120/gp41, synthesized as an inactive precursor, gp160. During its transit through the host cell constitutive secretory pathway, gp160 is cleaved at the conserved RE-KR↓AV sequence, generating the gp120/gp41 complex present at the surface of the infectious viruses [1].

Recently, seven members of a novel family of kexin/subtilisin-like serine proteinases known as pro-protein convertases (PCs) have been molecularly characterized. These include the widely expressed furin and PC7, the endocrine and non-endocrine enzymes PC5 and PACE4, the neural and endocrine

convertases PC1 and PC2 and the germ cell-specific PC4 (for reviews and updates see [2–5]). Because of their general preference for cleavage post specific single or pairs of basic residues, some of these enzymes have been proposed to participate in the processing of gp160. Thus, furin was first shown to cleave gp160 intracellularly into gp120/gp41 by Hallenberger et al. [6]. Cellular coexpression studies [7] and in vitro assays [8,9] further demonstrated that furin, PACE4, PC5-A and its isoform PC5-B, and PC1 may process gp160. Since the main HIV host cells in vivo are the CD4⁺ lymphocytes, derived cell lines were shown to express mainly mRNA coding for furin, PC7 [9] and PC5 [9,10], suggesting that these convertases may be the best physiological candidates for the proteolytic cleavage of gp160 in T4 lymphocytes. However, it is important to perform such experiments in primary cells and not just in cell lines. Furthermore, since HIV replication mostly occurs in activated T4 lymphocytes [11], it also becomes imperative to define the convertases expressed in both resting and activated human immune cells.

Using semi-quantitative RT-PCR, in this work, we defined the main candidate gp160 convertase mRNAs expressed in resting and activated human T4 lymphocytes obtained from healthy donors. The detection of PC7-specific transcripts in these cells led us to evaluate by ex vivo and in vitro assays the putative involvement of PC7 in the proteolytic cleavage of gp160.

2. Materials and methods

2.1. Isolation of T4 lymphocytes by negative selection and RT-PCR assays

Human lymphocytes were isolated from peripheral blood mononuclear cells (PBMC) prepared on a Ficoll-Paque (Pharmacia no. 17084003) discontinuous gradient by 40 min centrifugation at 1800 rpm and 20°C. Lymphocytes were collected at the Ficoll interface and washed 3 times in 1% (w:v) bovine serum albumin in phosphate-buffered saline (PBS-BSA), in order to remove the platelets. The PBMC resuspended at a density of 5×10^6 cells/ml in PBS-1% BSA were incubated for 1 h at 37°C on plastic dishes in order to eliminate adherent monocytes. The floating cells were centrifuged for 10 min at 1400 rpm, resuspended at 20×10^6 cells/ml in PBS-1% BSA and incubated for 45 min at 4°C with a cocktail of antibodies at 20 µg/ml (OKT8, 3C10, NN3, LM-2, LYM-1, and IV.3). After two washes in PBS-1% BSA, the cells were incubated for two rounds for 45 min at 4°C on a plastic petri dish precoated with anti-mouse IgG at 20 µg/ml (Sigma M8642) in 0.05 M Tris-HCl pH 9. The cells collected in the supernatant were assessed by fluorescence-activated cell sorting (FACS) analysis and shown to contain at least 96% of CD4⁺ cells in each experiment. The cells were then cultured for 4 days in an RPMI medium containing synthetic serum (ICN Flow), 1% bovine serum albumin, with or without [2 µg/ml phytohaemagglutinin (PHA), and 15 units/ml of interleukin-2 IL-2] (Gibco). The RT-PCR reactions

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Abbreviations: VV, vaccinia virus; PC, precursor convertase; RT-PCR, reverse transcriptase polymerase chain reaction; HIV, human immunodeficiency virus; gp, glycoprotein; WT, wild-type; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorting

were performed after TRIzol RNA extraction with the human PC-specific oligonucleotides, as described in [9]. The antibodies used were purified from the supernatant of hybridoma cultures using a T-gel column (Pierce). The hybridomas (ATCC) used produce mouse antibody reactive to CD4 receptors (OKT4; no. CRL-8002), CD8 receptors (OKT8; no. CRL-8014), Fc receptors (cd14) (3C10; no. TIB-228), glycophorin A (NN3; no. HB 8474), mac-1 receptor (cd11b) (LM/2; no. HB-204), B cells (LYM-1; no. HB-8612), natural killer and killer cells (HNK-1; no. TIB-200) and IgG Fc receptor II (cdw32) (IV.3; a gift from Dr. R. Sekaly, IRCM).

2.2. Vaccinia virus recombinants and cellular expression studies

The recombinant vaccinia virus (VV) encoding gp160 and VV:BTMD-hfurin were respectively gifts from Drs. B. Moss (NIH), and G. Thomas (Vollum Institute, Oregon). The purified vaccinia viruses recombinant expressing full length hfurin [12] and rat PC7 and its truncated form before its transmembrane domain (BTMD-rPC7) (S. Munzer and N.G. Seidah, submitted) were obtained as in [12]. Co-expression studies using VV:gp160 and either VV:hfurin or VV:rPC7, the 4 h [³⁵S]methionine labeling of BSC40 and AtT20 cells and the immunoprecipitation of gp160 and its processed products were performed as in [7].

2.3. Purification of convertases, fluorometric and in vitro assays

Secreted BTMD-hfurin and BTMD-rPC7 were purified from the media of VV infected GH₄C₁ cells using a previously described purification procedure [9]. Enzymatic activities were monitored using the fluorogenic substrate pERTKR-MCA [200 μM] in 1 mM CaCl₂, 50 mM Tris-acetate, pH 6.5 for BTMD-rPC7 and pH 7 for BTMD-hfurin as described [8]. In vitro endoproteolytic cleavage of gp160 and of the synthetic peptide spanning the gp120-gp41 junction was performed as in [9].

3. Results

3.1. Expression of convertases in freshly isolated human T4 lymphocytes

T4 lymphocytes, the major target of HIV, were purified by a negative panning technique from the blood of healthy donors. FACS analysis demonstrated that at least 96% of the isolated cells are CD4⁺ (not shown). Since HIV viral replication occurs mainly in activated T4 lymphocytes [11], using semi-quantitative RT-PCR we compared the expression of the PCs in both resting and T4 lymphocytes activated with a mixture of PHA and IL-2. Fig. 1 shows the relative mRNA levels of PCs in T4 lymphocytes, at days 0 and 4 with or without activation. The results were normalized with respect to the levels of mRNA expression of the ribosomal house-keeping protein L27, as described [9]. These data, averaged over duplicate results obtained from T4 cell preparations of 3 healthy individuals, demonstrate that PC7, PC5 and furin are the three main convertases expressed in resting T4 Lymphocytes. In contrast, no PC1 or PC2 mRNA transcripts were found in the isolated cells and only insignificant levels of PACE4 mRNA were detected in one of the 3 independent preparations. Furthermore, we demonstrated that culturing T4 lymphocytes for 4 days, especially in the presence of PHA/IL-2, leads to a significant increase in the level of expression of furin and PC7, but not of PC5 (Fig. 1). These data suggest that furin [6–9] and the newly discovered PC7 [5] represent the major PC-like candidates for the processing of gp160 in activated T4 lymphocytes.

3.2. Comparative processing of gp160 by furin and PC7 in BSC40 and AtT20 cells

Since we previously reported that furin cleaves gp160 to gp120/gp41 and gp120 into gp77/gp53 in both constitutively

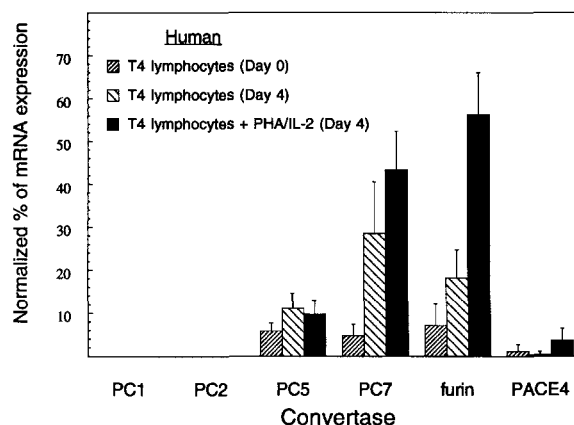
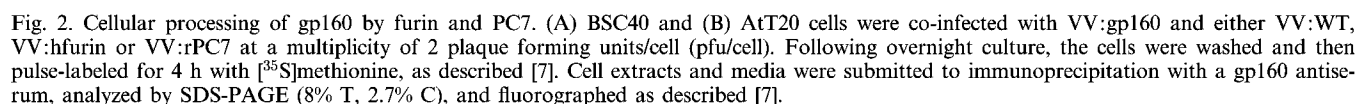


Fig. 1. Identification of precursor convertases mRNA in resting and 4-day activated human T4 lymphocytes from healthy donors. Human T4 lymphocytes were isolated (day 0) and cultured for 4 days in the absence or presence of a mixture of PHA and IL-2. Each RT-PCR amplification (28 cycles) was performed using convertase specific primers together with two others specific for the control ribosomal L27 protein, as described [9]. The bar graphs represent the quantitation of the relative amount of each convertase normalized to the control L27. The data are an average (\pm standard deviation) of RT-PCR experiments performed on RNA isolated from duplicate values obtained from 3 different individual donors.

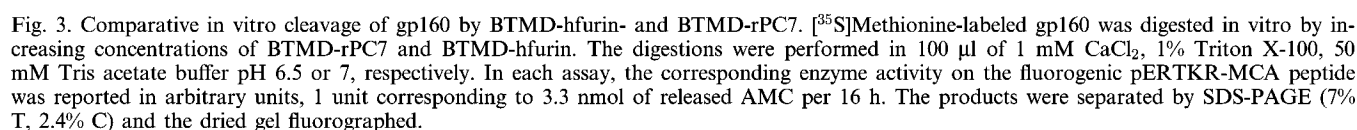
secreting, e.g. BSC40 cells and in regulated cells containing secretory granules, e.g. AtT20 cells [7], we compared the processing of gp160 by either furin or PC7 in both cell types. Thus, following overnight infections with either VV:gp160 and either VV:WT, VV:rPC7 or VV:hfurin, BSC40 (Fig. 2A) and AtT20 (Fig. 2B) cells were pulse-labeled with [³⁵S]methionine for 4 h. Cell extracts and media were analyzed by SDS-PAGE of the immunoprecipitated proteins using a gp160 antiserum derived from a pool of AIDS patients [7]. In the control experiment (VV:WT), we note the formation of some gp120 resulting from endogenous BSC40 or AtT20 convertase activities. Note that in AtT20 cells, the gp150 protein represents the initial ER-localized precursor, whereas gp160 is the convertase-susceptible form which reached the trans Golgi network [7]. In contrast, in BSC40 cells both forms comigrate using these SDS-PAGE separation conditions [7]. Differently from furin, the degree of increase in the level of gp120 secreted into the medium suggests that processing of gp160 into gp120/gp41 by PC7 is much more efficient in BSC40 cells as compared to AtT20 cells (Fig. 2). On the other hand, the generation of gp77/gp53 from gp120 [7,8] is best accomplished by furin in either cell type. Thus, we conclude that furin and PC7 can process gp160 into gp120, but that PC7 can best generate the latter product in constitutively secreting cells. In addition, further processing of gp120 into gp77/gp53 is mainly performed by furin. Interestingly, in AtT20 cells and to a lesser extent in BSC40 cells, rPC7 (89 kDa) co-immunoprecipitated with the HIV envelope products in cell extracts (Fig. 2). The same band was also detected with a PC7-specific antibody (not shown). Although we do not know the exact mechanism for this phenomenon, it is interesting to note that gp120 was recently shown to bind fibronectin [13], suggesting that gp120 can interact with proteins other than the CD4⁺ receptor or its co-receptor fusin [14].

3.3. In vitro digestion of gp160 by soluble rPC7 and hfurin

In order to compare the efficiency of gp160 processing by



using a recombinant vaccinia virus, and partially purified on a lentil-lectin column [8]. The source of soluble hfurin (BTMD-hfurin) and rPC7 (BTMD-rPC7) was the 50-fold con-



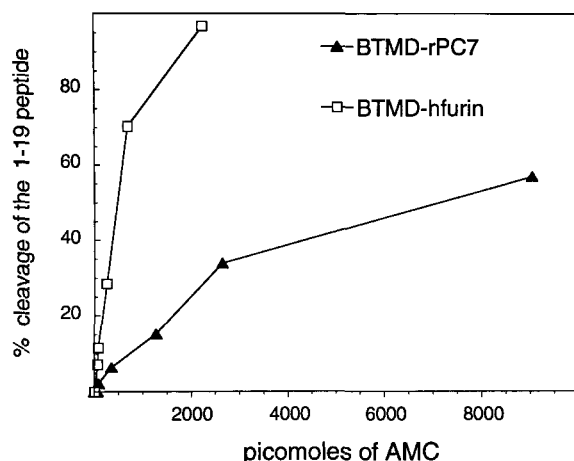


Fig. 4. Cleavage of the gp120/gp41 junctional 19 aa peptide by BTMD-hfurin and BTMD-rPC7. The 19 aa model peptide was incubated for 1 h at 37°C with increasing activities of either BTMD-hfurin or BTMD-rPC7, based on the level of AMC released from pERTKR-MCA. The generated peptides were separated on a Vydak C18 RP-HPLC column, their elution monitored at 214 nm, and the area under each peptide peak was integrated. The % cleavage is defined as the area under each product peak divided by the total areas.

centrate obtained from the media of GH₄C₁ cells infected with their VV:recombinants. In vitro digestions were performed using increasing concentrations of enzymes with 15 000 cpm of [³⁵S]methionine-labeled gp160. Fig. 3 demonstrates that using two comparable levels of enzymatic activity, measured in parallel with the fluorogenic pERTKR-AMC peptide, the soluble BTMD-hfurin is much more efficient in cleaving gp160 into gp120 and gp41 as compared to BTMD-rPC7, which barely cleaves gp160 in vitro. However, in BSC40 cells rPC7 cleaves gp160 much better than BTMD-rPC7 (not shown), suggesting that the membrane-anchored rPC7 (S. Munzer and N.G. Seidah, submitted) is more active on gp160 than the soluble BTMD form.

3.4. In vitro digestion of a model 19 amino acid peptide spanning the gp120/gp41 cleavage site

Since no cleavage kinetics could be derived from the gp160 processing experiment, we chose to analyze the specificity and kinetics of cleavage of the gp120/gp41 site by studying the processing of a model 19 amino acid peptide (PTKAKRRVVQREKR₅₁₂↓AVGIG) encompassing the sequence at the REKR-AV junction of gp120 and gp41. This model peptide was digested by either BTMD-hfurin and BTMD-rPC7 and the cleavage products were separated by RP-HPLC and characterized by amino acid analysis, as described [9]. Both furin and PC7 yield two main products (PTKAKRRVVQREKR and AVGIG, not shown), resulting from the cleavage of this peptide at the same position as the physiological gp160 in vivo cleavage site. Fig. 4 depicts the calculated percent of peptide cleaved by increasing activities of furin and PC7, measured with the substrate pERTKR-MCA. These data suggest that the 19 amino acid peptide is better cleaved by BTMD-hfurin than by BTMD-rPC7. In the absence of a specific active site titrant for the PCs, we performed kinetic measurements allowing us to estimate the K_m of this reaction for each enzyme. The data (not shown) revealed that the K_m deduced from the BTMD-rPC7 processing

is about $45 \pm 8 \mu\text{M}$, which is quite similar to that estimated for BTMD-hfurin ($16 \pm 7 \mu\text{M}$) [9]. Therefore, these data suggest that the difference in the ability of furin and PC7 to process gp160 in vitro may be due to a higher turnover rate (k_{cat} value) for soluble furin as compared to soluble PC7.

4. Discussion

The results presented in this work define the nature of the major convertases expressed in resting human T4 lymphocytes as furin, PC7 and PC5 and, in activated T4 lymphocytes as furin and PC7 (Fig. 1). These data resemble those derived from immortalized CD4⁺ cell lines, such as Jurkat, CEM-T4 and SupT-1 [9]. In agreement with previous data demonstrating that phorbol esters upregulate the level of furin in H9 cells [8], our results showed that activation of primary human T4 lymphocytes with a mixture of PHA and IL-2, known to enhance HIV viral replication [11], increases the level of furin and PC7 with little effect on PC5 (Fig. 1). However, we also note that just culturing T4 lymphocytes for 4 days without added PHA/IL-2 results in an upregulation of the level of PC7 mRNA but not that of furin (Fig. 1). These results also suggest that the mRNA level of PC7 is more sensitive than that of furin to effectors already present in the culture medium. It will be of interest to define the endogenous T4-cell substrates for these enzymes which may be co-regulated by the same activation and which when cleaved could perform critical functions in the immune system.

In view of the fact that the constitutively secreting T4 lymphocytes are the main targets of HIV infection, our analysis of PC-expression results suggest that furin and PC7 are the main PC-like candidate gp160 convertases. To test this hypothesis directly, we co-expressed vaccinia virus recombinants coding for gp160 and each of the above two enzymes. In an earlier study, we showed that furin processes gp160 intracellularly much better than PC5 [7] and, in this study, PC5 was demonstrated to be less expressed in activated lymphocytes than either furin or PC7. Thus, in the present report the ex vivo and in vitro gp160 processing by furin was compared to that obtained with PC7. Cellular co-expression results showed that furin and PC7 cleaved gp160 differently (Fig. 2). Thus, whereas PC7 mainly processes gp160 into gp120/gp41, especially in the constitutively secreting BSC40 cells, furin further cleaves gp120 to gp77/gp53, as previously reported [7]. In a similar fashion, PC7 was also shown to cleave the PC-inhibitor protein $\alpha 1$ -PDX [8,9,15] much better in constitutive versus regulated cells (S. Benjannet et al., submitted). A possible explanation for the cell-type specificity observed for PC7 may be related to the fact that this enzyme is mostly localized in the Golgi apparatus in either cell types (N.G. Seidah and M. Marcinkiewicz, unpublished data), whereas furin and gp160 may transit from the immature granules of AtT20 cells to the cell surface and into endosomes [16,17].

In order to define the cleavage kinetics of gp160 into gp120/gp41 by either enzyme, we compared the ability of soluble BTMD-hfurin and BTMD-rPC7 to cleave in vitro [³⁵S]methionine-labeled gp160 (Fig. 3). This experiment demonstrated that for equal enzymatic activity (measured on the pERTKR-MCA peptide) BTMD-hfurin cleaves gp160 into gp120/gp41 much more efficiently than BTMD-rPC7. These in vitro results contrast with those obtained in BSC40 cells (Fig. 2) which demonstrated that the membrane-anchored

PC7 (in contrast to BTMD-rPC7) is able to efficiently cleave gp160 into gp120/gp41 intracellularly. Thus, it is possible that membrane anchoring of either native PC7 or furin may favor the interaction of PC7 with the membrane-bound gp160. Alternatively, rPC7 may interact with other cellular proteins for maximal activity or it may activate yet another undefined gp160 processing enzyme, as previously suggested [18,19].

Finally, in order to derive more precise kinetic values for this cleavage, furin and PC7 were compared in their ability to process a 19 aa model peptide spanning the gp120/gp41 junction [9]. The data demonstrated that BTMD-rPC7 cleaves this peptide at the expected REKR↓AV physiological site with a K_m of $45 \pm 8 \mu\text{M}$ as compared to that of BTMD-hfurin estimated at $16 \pm 7 \mu\text{M}$. This difference in K_m values does not, by itself, explain the marked efficiency of soluble furin as compared to BTMD-rPC7 to cleave either gp160 or the 19 aa peptide (Figs. 3 and 4). It is thus likely that the turnover number (k_{cat}) of BTMD-hfurin is higher than that of BTMD-rPC7. However, in the absence of active site titrant, the answer to this question will have to await further experiments.

In conclusion, the data presented demonstrate that both furin and PC7 are the main candidate PC-like enzymes which could participate in the processing of gp160 in T4 lymphocytes activated under conditions favoring HIV replication. It is interesting to note that a recent report suggested that a PACE4-activated protein with an apparent molecular mass of about 90–95 kDa (similar mass to PC7) was expressed in the furin-deficient RPE-40 cells, in which gp160 is efficiently cleaved into gp120/gp41 [16]. Since both furin and PC7 require $< 100 \mu\text{M}$ Ca^{2+} for activity, we found it very difficult to eliminate their membrane-bound activity by using chelators such as EDTA or EGTA at 1–2 mM concentrations (J.C. Munzer and N.G. Seidah, submitted). Thus, future work should clarify the degree of involvement in gp160 processing of furin and PC7 in cell types other than the T4 lymphocytes and whether truly calcium-independent enzymes, distinct from PC-like convertases [18,19], also participate in the *in vivo* processing of gp160.

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